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(54) Bone reinforcing agent and foods and drinks product containing the same

(57) A bone reinforcing agent comprising a basic protein fraction or a basic peptide fraction derived from milk as an effective component. The basic protein fraction is obtained preparing milk or a raw material derived from milk in a cation exchange resin and eluting the adhered fraction. The basic peptide fraction is obtained by hydrolyzing the basic protein fraction with a protease. The basic protein fraction and basic peptide fraction of the present invention promote growth of osteoblast and suppress resorption of osteoclast, and thereby strengthen bones, when orally administered. It is useful for treating or preventing various bone diseases such as osteoporosis.

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Description

The present invention relates to a bone reinforcing agent and food and drink products containing the same exhibiting a bone reinforcing activity. Because the bone reinforcing agent and the food and drink products containing the same of the present invention exhibit the effects of promoting the growth of osteoblast and suppressing bone resorption by osteoclast, they are useful in treating or preventing various bone diseases such as osteoporosis, bone fractures, rheumatism, and arthritis.

In recent years, various bone diseases such as osteoporosis, bone fractures, lumbago, and the like, are increasing along with the progressive increase in the elderly population. These diseases are caused by lack of calcium intake, lowering of calcium absorbing ability, imbalance of hormones after postmenopause, and the like. Increasing the peak bone mass, which is the amount of bone in the body, is considered to be effective in preventing bone diseases such as osteoporosis, bone fractures, lumbago, and the like in aged people. To increase the peak bone mass is nothing other than to strengthen the bone. To control bone resorption is also considered to be effective in preventing osteoporosis. Bones always repeat a balanced formation-resorption cycle which is called remodeling. Imbalance in hormones after postmenopause causes bone resorption to predominate over bone formation, and induces osteoporosis. Accordingly, bones are reinforced by controlling bone resorption and maintaining the amount of bone at a certain level.

Various calcium agents, such as calcium salts (e.g. calcium carbonate, calcium lactate, calcium phosphate), milk or whey calcium, and natural calcium agents (e.g. cattle bone meal, egg shell), and the like, are used to strengthen the bones. These are individually administered or added to food or drink together with other additives which have the effect of increasing calcium absorption, such as casein phosphopeptide. However, more than half of the calcium salts and natural calcium administered are said to be excreted without being absorbed in the body. Even if absorbed, calcium may not necessarily be utilized for the improvement of bone metabolism or the reinforcement of bones because the affinity of calcium to bone differs according to the form of the calcium and types of other nutrients which are taken together with the calcium. Vitamin D_3, calcitonin preparations, estrogen preparations, and the like are known as drugs for treating osteoporosis or reinforcing bones. In addition, new drugs such as bisphosphonate preparations are under development. Administration of these drugs, however, may be accompanied by side effects such as ear noises, headache, and anorexia. Furthermore, the addition of these drugs to food or drink is currently infeasible from the aspect of safety, cost, and the like. Therefore, because of the nature of osteoporosis, the development of a bone reinforcing agent, or a food or drink product containing a bone reinforcing agent, which can be orally administered over an extended period of time and which directly exhibits the bone growth promoting effect or the bone resorption suppressing effect, and is effective in the treatment or prevention of the osteoporosis, has been desired.

In view of the above-mentioned problems, the present inventors have undertaken extensive research into the substances contained in the various raw materials for foods which exhibit a bone reinforcing effect. This research has resulted in the finding that a basic protein fraction derived from milk or basic peptide fractions obtained by hydrolyzing the basic protein fraction with a protease, such as pepsin or pancreatin, exhibit the effects of promoting growth of osteoblast and suppressing resorption of osteoclast, and can strengthen bone when orally administered. The inventors of the present invention have found that the basic protein fraction and the basic peptide fraction can be used as a bone reinforcing agent or as an effective component for bone reinforcing food and drink. These findings have led to the completion of the present invention.

SUMMARY OF THE INVENTION

Accordingly, the present invention can provide a bone reinforcing agent,and a food or drink product containing the same, exhibiting the effects of promoting growth of osteoblast and suppressing resorption of osteoclast, thereby strengthening bone without causing side effects.

This can be attained by a bone reinforcing agent, or a food or drink product containing the same, which contains a basic protein fraction derived from milk or a basic peptide fraction obtained by hydrolyzing this basic protein fraction with a protease.

Specifically, the present invention relates to a bone reinforcing agent which contains a basic protein fraction derived from milk or a basic peptide fraction, obtained by hydrolyzing this basic protein fraction with a protease, as an effective component.

The present invention further relates to a bone reinforcing food or drink product which contains the basic protein fraction, or the basic peptide fraction, as an effective component.

Other objects, features and advantages of the invention will hereinafter become more readily apparent from the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the osteoblast growth promoting activity of the basic protein fraction of the present invention in Test Example 5.

Figure 2 shows the osteoblast growth promoting activity of the basic protein fraction and the basic peptide fraction of the present invention in Test Example 6.

Figure 3 shows the osteoclast bone resorption suppressing activity of the basic protein fraction and the basic peptide fraction of the present invention in Test Example 6.

Figure 4 shows the effect of increasing the breaking force of femora by the basic protein fraction of the present invention in Test Example 7.

Figure 5 shows the growth promoting effect of tibiae epiphysial growth plates by the basic protein fraction of the present invention in Test Example 7.

Figure 6 is an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of the basic protein fraction of the present invention in Test Example 1.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The feature of the present invention is to provide a basic protein fraction derived from milk, or a basic peptide fraction obtained by hydrolyzing the basic protein fraction with a protease, as an effective component for the bone reinforcing agent or the food or drink containing the same. The basic protein fraction can be obtained from milks of mammals, such as cow milk, human milk, goat milk, sheep milk, and the like. The basic peptide fraction can be obtained by hydrolyzing the basic protein fraction with a protease. These fractions act directly on the bone to exhibit a bone reinforcing effect and a bone resorption suppressing effect, and strengthen the bone.

As later described in detail in the Test Examples 1-4, the basic protein fraction derived from milk has the following characteristics:

1) It comprises several proteins having a molecular weight in the range of 3,000-80,000 by SDS-PAGE.
2) It consists of 95% or more of the proteins and small amounts of fatty acids and ash components.
3) The major proteins are lactoferin and lactoperoxidase.
4) 15% or more of the amino acids for the proteins are basic amino acids such as lysine, histidine, arginine, and the like.

The basic protein fraction can be obtained, for example, by contacting a raw material derived from milk such as skim milk or whey to a cation exchange resin in order to adhere basic proteins, eluting the adhered basic protein fractions with an eluent with a salt content of 0.1-1.0 M, desalting and concentrating the collected fractions using a reverse osmosis (RO) membrane or by electrodialysis (ED) and, optionally, drying the desalted and concentrated product.

Other methods known in the art for obtaining the basic protein fraction include a method whereby milk or the raw material derived from milk is adhered in the cation exchange resin by contacting the former to the cation exchange resin and eluting this adhered basic protein fraction with an eluent with a pH of 5 or greater and an ion strength of 0.5 or greater (Japanese Patent Application Laid-open (kokai) No. 202098/1993); a method using arginic acid gel (Japanese Patent Application Laid-open (kokai) No. 246198/1986); a method of obtaining the basic protein fraction from whey using inorganic porous particles (Japanese Patent Application Laid-open (kokai) No. 86839/1989); a method of obtaining the basic protein fraction from milk using a sulfated ester (Japanese Patent Application Laid-open (kokai) No. 255300/1988); and the like. Any basic protein fraction obtained by any one of these methods can be used in the present invention.

The basic peptide fraction derived from milk has the same amino acid composition as the basic protein fraction, and can be obtained as a peptide composition with an average molecular weight of 4,000 or less by hydrolysis of the basic protein fraction with a protease such as pepsin, trypsin, chymotrypsin, or the like, and further, optionally, with other protease such as pancreatin or the like.

The basic protein fraction or the basic peptide fraction, which is the effective component of the bone reinforcing agent of the present invention, may be administered as it is or in suitable forms such as powder, granules, tablets, capsules, drinks, and the like by a conventional method. Furthermore, it is possible to incorporate the basic protein fraction or the basic peptide fraction, as it is or after it has been prepared in suitable forms, in nutrients, drinks, or foods, to strengthen the bones by promoting bone formation or suppressing bone resorption. Because the milk-derived basic protein fraction and the basic peptide fraction of the present invention are comparatively stable with respect to heat, it is possible to sterilize the raw materials containing these fractions with heat by a conventional method.

The dose of the basic protein fraction or the basic peptide fraction of the present invention depends on the age, the effects expected from the treatment, and the disease conditions. Tests using rats confirmed that the amount of the basic protein fraction or the basic peptide fraction for exhibiting the bone reinforcing effect is 0.1% by weight or more in feed.

Accordingly, the bone reinforcement effect can be expected by administering the basic protein fraction or the basic peptide fraction at a dose of 0.5 g/day of more to an adult, who generally takes 500 g/day on a dry basis of food and drink.

Because the bone reinforcing agent and the food containing the same of the present invention promote bone formation and suppress bone resorption, the bones are reinforced if these are administered. Accordingly, bone reinforcing agent and the food containing the same are useful for treating or preventing various bone diseases, such as osteoporosis, bone fractures, rheumatism, and arthritis. These are particularly effective in treating or preventing osteoporosis. Further, it is possible to increase the peak bone mass in the growth period by administering the bone reinforcing agent or the foods and drinks containing the same of the present invention to infants and children.

Example 1

A column (diameter: 5 cm, height: 30 cm), packed with 400 g of a cation exchange resin, sulfonated chytopal (trademark, manufactured by Fuji Spinning Co., Ltd.) was thoroughly washed with deionized water. 40 l of unsterilized skim milk (pH 6.7) was passed through this column at a flow rate of 25 ml/min, after which the column was thoroughly washed with deionized water. The basic protein fraction adhered in the resin was eluate with a 0.02 M carbonate buffer (pH 7.0) containing 0.98 M sodium chloride. The eluate was desalted and concentrated with an RO membrane and freeze-dried to obtain 21 g of powder of the basic protein fraction.

Test Example 1

The molecular weight of the basic protein fraction obtained in Example 1 was measured by SDS-PAGE and it was found that the molecular weight was distributed in the range of 3,000-80,000. The results are shown in Figure 6.

Test Example 2

The composition of the basic protein fraction prepared in Example 1 was analyzed. The results are shown in Table 1. These results indicate that almost all components in the fraction were proteins.

TABLE 1

	(wt.%)
Water	1.06
Proteins	96.50
Fats	0.56
Ash	0.27
Others	1.61

Test Example 3

The composition of the proteins in the basic protein fraction prepared in Example 1 was analyzed. The results are shown in Table 2. These results indicate that the basic protein fraction contains 40 wt.% or more of lactoferin and 40 wt.% or more of lactoperoxidase.

TABLE 2

	(wt.%)
Lactoferin	42.5
Lactoperoxidase	45.6
Insulin-like growth factor-I factor-I	0.005
Others	11.895

Test Example 4

The basic protein fraction obtained in Example 1 was hydrolyzed with 6 N hydrochloric acid at 110°C for 24 hours and analyzed by an amino acid analyzer (L-8500, trademark, manufactured by Hitachi, Co. Ltd.). The results are shown in Table 3. These results indicate that the basic protein fraction contain 15 wt.% or more of basic amino acid in the total amino acids.

TABLE 3

	(w%)
Aspartic acid	10.1
Serine	5.3
Glutamic acid	12.3
Proline	4.7
Alanine	5.7
Leucine	10.2
Lysine	8.4
Histidine	2.5
Arginine	7.2
Others	33.6

Example 2

A column (diameter: 100 cm, height: 10 cm), packed with 30 kg of a cation exchange resin, SP-Toyopal (trademark, manufactured by Toso Co. Ltd.) was thoroughly washed with deionized water. A cheese whey, sterilized by heating at 121°C for 30 seconds, was passed through this column at a flow rate of 10 l/min, after which the column was thoroughly washed with deionized water. The basic protein fraction adhered in the resin was eluted with a 0.1 M citrate buffer (pH 5.7) containing 0.9 M sodium chloride. The eluate was desalted and concentrated using the ED method and freeze-dried to obtain 183 g of powder of the basic protein fraction.

Test Example 5

The osteoblast growth promoting activity was tested on the basic protein fraction obtained in Example 1.

A stock of osteoblast (MC3T3-E1) for the cultivation was scattered over a 96-well flat bottom petri dish and cultured for 18 hours in an α-MEM culture medium (produced by Flow Laboratories Inc.) containing 0.2% calf serum. For the culture, 2 µl of a solution prepared by dissolving the basic protein fraction obtained in Example 1 at a concentration of 0.5% was added to 100 µl of the medium. After culturing, [methyl-^{3}H]-thymidine was added to measure the osteoblast growth promoting activity by measuring the radioactivity of the [methyl-^{3}H]-thymidine in the cells after two hours (Protocol for New Experiments of Cell Technology, pp 319-320 (1993), The University of Tokyo, Medical Scientific Research Center, Cancer Research Institute). The results of the experiment are shown in Figure 1, in which the cell growth promoting activity is indicated by the radioactivity (in percent) of the medium to which the basic protein fraction was added as specific activity, taking the radioactivity of the medium to which no basic protein fraction had been added as 100%. The Figure 1 shows that the osteoblast cell growth promoting activity in samples to which the basic protein fraction obtained in Example 1 or 2 added was almost twice that of the sample to which no basic protein fraction had been added.

Test Example 6

The osteoblast growth promoting activity and the effect of suppressing osteoclast resorption were investigated for (i) the basic protein fraction prepared in Example 2, (ii) the basic peptide fraction obtained by the hydrolysis of the basic protein fraction prepared in Example 1 with pepsin, and (iii) the basic peptide fraction obtained by the hydrolysis of the basic protein fraction prepared in Example 1 with pepsin and pancreatin, according to the everted gut suc method (Shiroh Gotoh et al. Nutrition Experiments with Small Animals, pp 83-85 (1980).

The small intestine was extirpated from a mature rat, after overnight fasting, and 7 cm of the duodenum from the pyloric region of the stomach was turned inside out. A Ringer solution was injected into this everted gut suc and then ligated. The everted gut suc was then incubated in an external solution in an external solution made up of the Ringer solution (control), or (a) the Ringer solution and 1% of the basic protein fraction prepared in Example 1, (b) the Ringer solution and 1% of the basic peptide fraction obtained by hydrolysis of the basic protein fraction prepared in Example 1 with pepsin, or (c) the Ringer solution and 1% of the basic peptide fraction obtained by hydrolysis of the basic protein fraction prepared in Example 1 with pepsin and pancreatin, and incubated at 37°C while oxygen gas was bubbled through the solution. After one hour, liquid was collected from the inside of each everted gut suc to determine the osteoblast growth promoting activity in the same manner as in Test Example 5.

The Femora were extirpated from rabbits (age: 10 days) and the soft tissues were removed. All the bone marrow cells containing osteoclast, prepared by mechanically pulverizing the femora in a medium containing 5% FBS, were

scattered over a piece of ivory in the amount of 200,000 cells/ml. 10% of a solution prepared by diluting the liquid collected from the insides of each everted gut suc three-fold, and the cells were cultured for two days. Bone resorption pits created on the ivory were stained with hematoxylin and the number was counted, to determine the effect of suppressing the osteoclast resorption (Kanji Seno, et al. The Manual for Animal Culture Cells for Different Research Themes, pp 199-200 (1993)).

The results are shown in Figures 2 and 3. In Figure 2, the effect of the osteoblast cell growth activity is indicated in terms of the radioactivity (in percent) relative to the radioactivity (100%) of the group for which the liquid was collected from the insides of each everted gut suc incubated in the Ringer solution and held for one hour (control). In the same manner, in Figure 3, the effect of suppressing osteoclast resorption is represented by the number of pits relative to the number of pits of the group for which the liquid was collected from the insides of each everted gut suc incubated in the Ringer solution and held for one hour (control).

Higher osteoblast growth promoting activities and higher effects of suppressing the osteoclast resorption were confirmed in all groups using (a) Ringer solution and 1% basic protein fraction prepared in Example 1, (b) Ringer solution and 1% basic peptide fraction obtained by the hydrolysis of the basic protein fraction prepared in Example 1 with pepsin, or (c) Ringer solution and 1% basic peptide fraction obtained by the hydrolysis of the basic protein fraction prepared in Example 1 with pepsin and pancreatin, as the external solutions for the everted gut suc, as compared with those of the group in which only the Ringer solution was used as the external solution. These results confirmed that the effective component of the bone reinforcing agent of the present invention can pass through the gastrointestinal tract.

Test Example 7

The bone reinforcing effect was tested on the basic protein fraction obtained in Example 2 by experiments using animal.

SD strain female rats (age: 6 weeks) were used for the experiment. The rats were fed preliminary for 1 week before the ovarieoctomized operation. Then, the animals were fed with a calcium defficient diet for 5 weeks. The osteoporosis was apparently induced in the rats fed with the calcium defficient diet after ovariectomy. These osteoporosis-induced rats were grouped into (A) a control group, (B) 0.1 wt.% basic protein fraction dosing group, (C) 0.5 wt.% basic protein fraction dosing group, (D) 1.0 wt.% basic protein fraction dosing group, each group consisting of 6 rats. Animals of each group were fed for 4 weeks with the test diets shown in Table 4. All diets were adjusted with casein so as to contain an equivalent amount (17.06%) of nitrogen. In addition, 300 mg of calcium, 230 mg of phosphorous, and 50 mg of magnesium were added to 100 g each diets.

TABLE 4

	(wt.%)			
	A	B	C	D
Casein	20.0	19.9	19.4	18.9
Corn starch	15.0	15.0	15.0	15.0
Cellulose	5.0	5.0	5.0	5.0
Corn oil	5.0	5.0	5.0	5.0
Vitamins	1.0	1.0	1.0	1.0
Minerals	2.65	2.65	2.65	2.65
Sucrose	51.05	51.05	51.15	51.15
DL-Methionine	0.3	0.3	0.3	0.3
Basic protein fraction	-	0.1	0.5	1.0

After 4 weeks, the both femora and tibiae were extirpated. The breaking force of femora was measured by the bone fracture properties measuring device (Rheometer Max RX-1600, trademark, manufactured by Aitekno Co. Ltd.). The tibiae were electrically demineralized and stained with Hematoxylin-Eosin according to measure the length of the epiphysial growth plate. The results are shown in Figures 4 and 5. The groups to which the basic protein fraction was administered (groups B to D) were confirmed to exhibit a larger breaking force of femore than the control group (group A). Further, the greater the concentration of basic protein fraction, the greater the values of femora breaking strength. Also, the length of tibia epiphysial growth plate was significantly and dose-dependently longer in the groups to which the basic protein fraction was administered (groups B to D) than in the control group (group A).

Example 3

A bone reinforcing drink of the composition shown in Table 5 was prepared.

TABLE 5

	(wt.%)
Mixed isomerized sugar	15.0
Fruit juice	10.0
Citric acid	0.5
Powder of basic protein fraction (Example 1)	0.5
Spice	0.1
Calcium	0.1
Water	73.5

Example 4

A paste with a composition shown in Table 6 was formed and baked to prepare bone reinforcing biscuit.

TABLE 6

	(wt.%)
Wheat	50.0
Sugar	20.0
Salt	0.5
Margarine	12.5
Egg	12.5
Water	2.5
Sodium bicarbonate	0.15
Ammonium bicarbonate	0.2
Calcium carbonate	0.45
Powder of basic protein fraction (Example 1)	1.2

Example 5

Tablets of bone reinforcing agent with a composition shown in Table 7 were prepares.

TABLE 7

	(wt.%)
Hydrous crystalline glucose	73.5
Fraction prepared in the Example 2	20.0
Calcium	5.0
Sugar ester	1.0
Perfume	0.5

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

Claims

1. A bone reinforcing agent comprising a basic protein fraction derived from milk as an effective component.

2. The bone reinforcing agent according to claim 1, wherein the basic protein fraction derived from milk has an amino

acid composition containing 15% by weight or more of basic amino acids.

3. The bone reinforcing agent according to claim 1 or claim 2, wherein the basic protein fraction derived from milk contains 95% by weight or more of proteins which mainly consist of lactoferin and lactoperoxidase and have a molecular weight of 3,000-80,000 Dalton as measured by SDS-PAGE.

4. The bone reinforcing agent according to claim 1, wherein the basic protein fraction derived from milk is obtained by contacting milk or a raw material derived from milk to a cation exchange resin to adhere the basic protein fraction and eluting the adhered fraction with an eluent having a base concentration of 0.1 M to 1.0M.

5. A bone reinforcing agent comprising, as an effective component, a basic peptide fraction which is obtained by hydrolyzing the basic protein fraction derived from milk, defined in any one of claims 1-4, with a protease.

6. The bone reinforcing agent according to claim 5, wherein the basic peptide fraction has an average molecular weight of 4,000 Dalton or smaller.

7. The bone reinforcing agent according to claim 5 or claim 6, in which the protease is at least one protease selected from pepsin, trypsin, and chymotrypsin.

8. The bone reinforcing agent according to any one of claims 5-7, wherein the protease is pancreatin and at least one protease selected from pepsin, trypsin, and chymotrypsin.

9. The bone reinforcing agent according to any one of claims 1-8, which exhibits effects of promoting growth of osteoblast and suppressing resorption of osteoclast.

10. A food or drink composition comprising the basic protein fraction derived from milk defined in any one of claims 1-4 or the basic peptide fraction defined in any one of claims 5-7, or both.

Fig.1

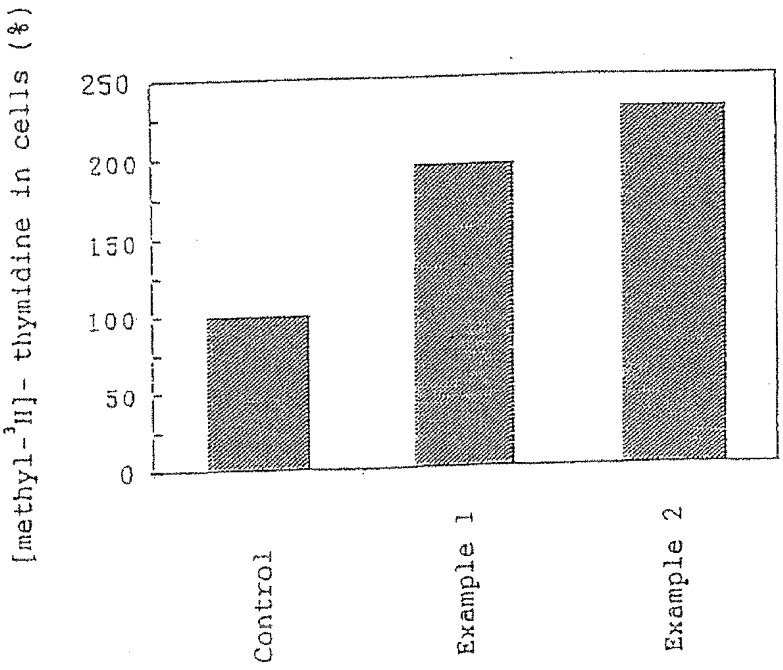


Fig.2

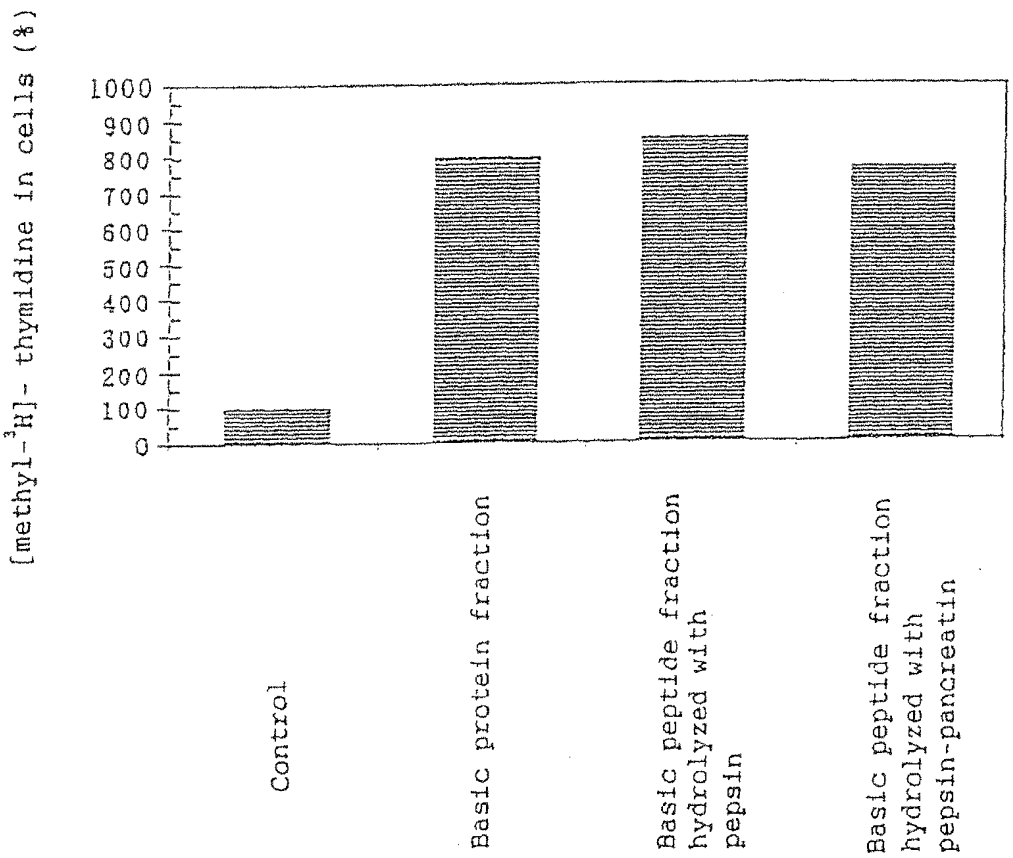


Fig.3

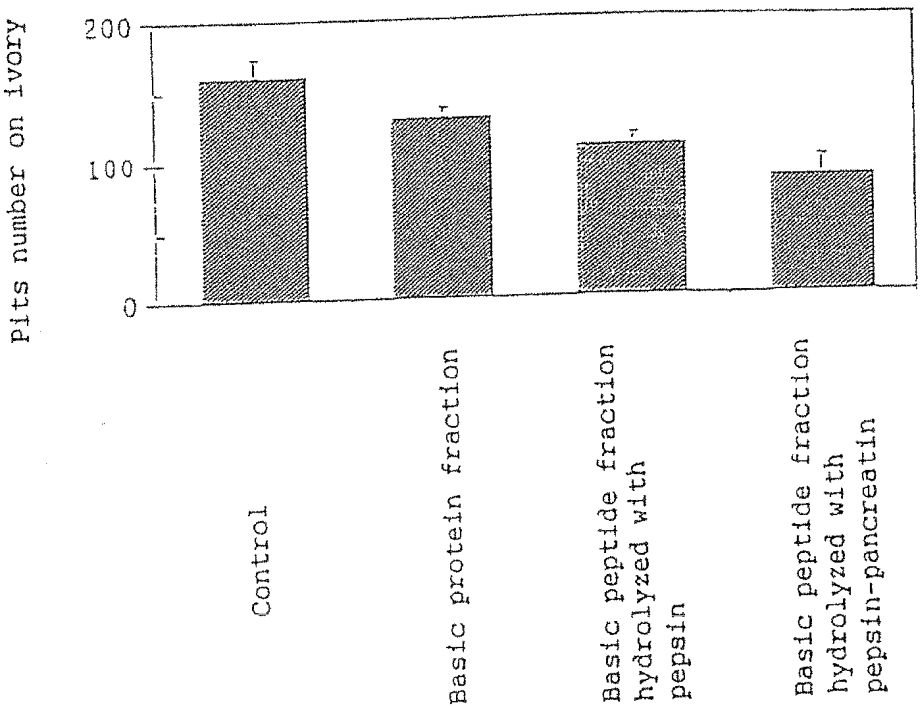


Fig.4

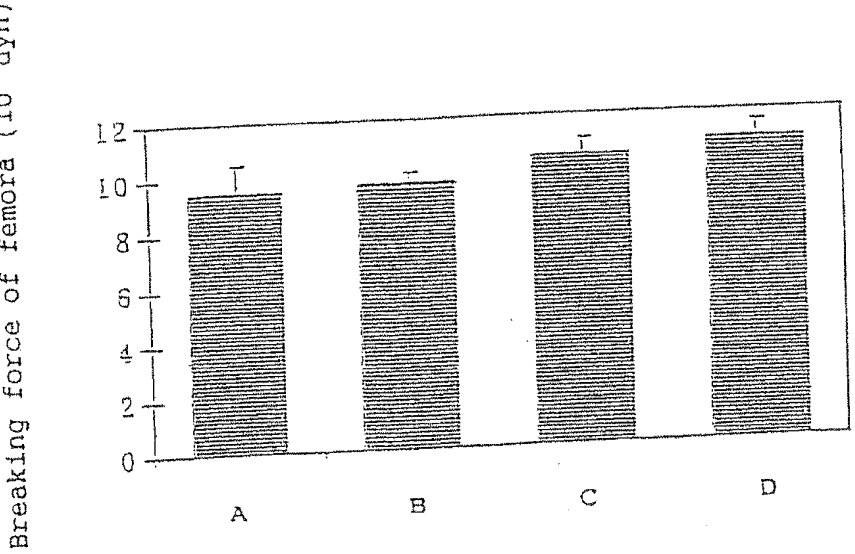


Fig.5

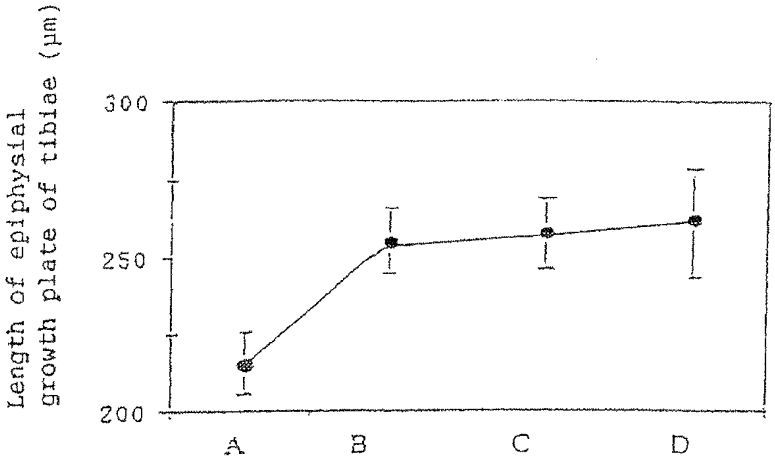
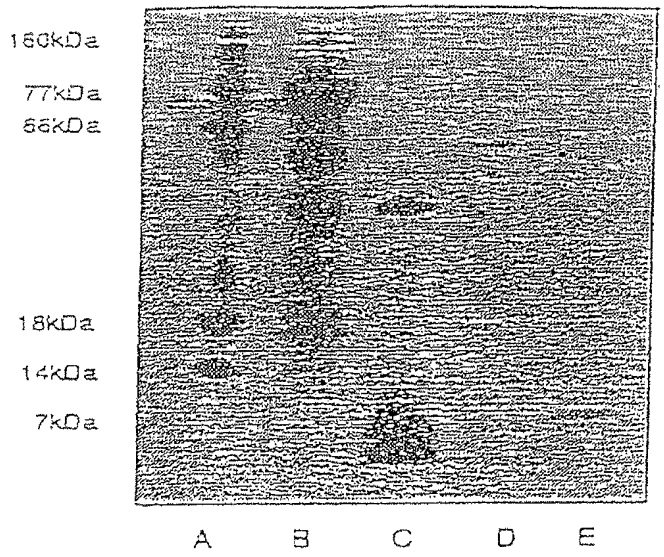


Fig.6



Pattern of SDS-PAGE

A; Marker
B; Basic protein fraction
C; hydrolysate of B with pepsin
D; hydrolysate of B with pepsin-pancreatin
E; Marker